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Fluxapyroxad Haptens and Antibodies for Highly Sensitive Immunoanalysis of Food Samples

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Abstract

Fluxapyroxad is a new-generation carboxamide fungicide, with residues increasingly being found in food samples. Immunochemical assays have gained acceptance in food quality control as rapid, cost-effective, sensitive, and selective methods for large sample throughput and in situ applications. In the present study, immunoreagents to fluxapyroxad were obtained for the first time, and competitive immunoassays were developed for the sensitive and specific determination of fluxapyroxad residues in food samples. Two carboxyl-functionalized analogues of fluxapyroxad were prepared and antibodies with IC_{50} values in the low nanomolar range were generated from both haptens, though a dissimilar response was observed concerning specificity. A robust direct assay was set up, with a calibration curve exhibiting a limit of detection of 0.05 nM (0.02 μg/L). Limits of quantitation of 5 μg/L were obtained for peach, apple, and grape juices using samples diluted in water. The direct immunoassay was also successfully applied to the determination of fluxapyroxad in grapes from in-field treated grapevines.

Keywords

Fungicide, ELISA, antibody, food safety, chemical residue

INTRODUCTION

Fluxapyroxad is a new-generation broad-spectrum fungicide that belongs to the pyrazole carboxamide class of active biocides. It stunts spore germination as well as germ tube and mycelium growth in the target species via inhibition of the succinate dehydrogenase (SDH) activity in complex II of the mitochondrial respiratory chain, which catalyses the oxidation of succinate to fumarate by reducing ubiquinone to ubiquinol. This enzyme is involved in two essential metabolic pathways, *i.e.*, the oxidative phosphorylation and the Krebs cycle, which makes SDH an excellent target for fungi control. This particular and effective mode of action converts SDH inhibitors into outstanding candidates for co-formulation with other fungicides in order to prevent the emergence of resistance.[1–4] The 2014 global market for SDH-inhibitor fungicides was valued at $2721 million, and it is expected to grow at a compound annual growth rate of 7.1% to mature into a $4046.6 million market by 2020.[5]

Fluxapyroxad (Figure 1) was recently developed on the basis of the molecular structure of boscalid, the most successful SDH inhibitor to date, by systematically modifying different parts of the molecule.[6] It displays low acute toxicity to humans by the oral, dermal, and inhalation routes; it is not irritating to the eyes and skin and is classified as “not likely to be carcinogenic”. The LD_{50} in rats treated orally or dermally with fluxapyroxad was greater than 2000 mg/kg of body weight. Low harm to terrestrial organisms was found; however, acute risk to estuarine/marine species could occur if formulations of fluxapyroxad were applied in areas that may result in spray drift to such environments.[7–9] Because of its environmental and health safety, as well

as its prominent pesticide efficiency, fluxapyroxad and other SDH inhibitor fungicides are deemed outstanding candidates for fungal disease control in the near future.

Gas and high-performance liquid chromatography coupled to different sorts of detectors are the most common analytical methods that are employed nowadays for chemical residue as well as contaminant detection. These are extremely sensitive and selective techniques, especially when coupled to mass spectrometers, and constitute the methods of choice for large monitoring programmes for which an extraordinary multiresidue capacity is demanded.[10] Nevertheless, other analytical applications, such as toxicological crisis, industrial control departments, dissipation and transfer studies, or just personal assessment, require straightforward, low cost, and rapid procedures for which the immunochemical methods provide a powerful alternative approach.[11-13] Antibody-based techniques are very selective and highly sensitive, and can be developed in a variety of immunoassay formats. Undoubtedly, the competitive enzyme-linked immunosorbent assay (cELISA) is the most common immunochemical method, probably due to its large sample throughput as well as its ability to provide quantitative results.

In order to develop a novel cELISA procedure, three immunoreagents are needed: the immunizing conjugate, the antibody, and the assay conjugate. The two required conjugates generally consist of a protein to which a functionalized derivative mimicking the target analyte, called a hapten, is linked. This synthetic molecule is usually prepared with a spacer arm holding a functional chemical group for covalent coupling. Rational design of different functionalized derivatives constitutes a key step for high-affinity and specific antibody generation as well as immunoassay

development.[14] Haptens that are employed for the preparation of immunizing conjugates should preferentially display the characteristic moieties and preserve the electronic distribution and conformation of the target compound.[15] However, the optimum linker tethering site is usually difficult to predict, and intricate synthetic procedures are most commonly required in order to prepare alternative immunizing haptens.[16] On the other hand, it is well established that employing heterologous haptens, i.e. molecules that are different from the immunizing hapten, in cELISA can improve the sensitivity of the assay and even modulate the selectivity of polyclonal antibody-based immunoassays.[17,18] Hapten heterologies most commonly consist of changes in the immunodeterminant moieties of the target molecule or a different linker composition or tethering site. These strategies have been shown to be quite effective, particularly in the conjugate-coated assay format.[19–21] Nevertheless, further studies are required aiming to increase the scientific knowledge about the structure–immunogenicity and structure–assay activity relationships of haptens.

In this study, we developed for the first time high-affinity antibodies specific to fluxapyroxad. In order to reach this goal, we investigated the influence of two proximate linker tethering sites over the generation of antibodies and their use as heterologous haptens. With this aim, polyclonal antibodies were raised since they are better representatives of the immune response. Finally, alternative cELISA procedures were developed, and their applicability to the analysis of residues of this agrochemical in food samples was evaluated.

MATERIALS AND METHODS

Reagents and Instruments. Technical fluxapyroxad was generously provided by BASF (Ludwigshafen, Germany) and Pestanal grade of fluxapyroxad and other pesticide standards were purchased from Sigma/Aldrich (Madrid, Spain). Compounds employed in this work only present slight safety concerns. Nevertheless, working in a well-ventilated fume hood is advisable for synthesis work. Bovine serum albumin (BSA) fraction V was from Roche Applied Science (Mannheim, Germany). Ovalbumin (OVA), horseradish peroxidase (HRP), and adult bovine serum (ABS) were purchased from Sigma/Aldrich (Madrid, Spain). Sephadex G-25 HiTrap Desalting columns (GE Healthcare, Uppsala, Sweden) were utilized for protein–hapten conjugate purification. Goat anti-rabbit immunoglobulin polyclonal antibody conjugated to peroxidase was from BioRad (Madrid, Spain). Costar flat-bottom high-binding 96-well polystyrene ELISA plates were from Corning (Corning, NY). UV–Vis spectra of haptens and conjugates as well as ELISA absorbances were read with a PowerWave HT from BioTek Instruments (Winooski, VT). Microwells were washed with an ELx405 microplate washer also from BioTek Instruments.

Preparation of Hapten FX*n*. The schematic representation of the synthesis of hapten FX*n* is depicted in Figure 2.

Synthesis of ethyl 1-allyl-3-(difluoromethyl)-1H-pyrazole-4-carboxylate, **2**. K_2CO_3 (278 mg, 2.012 mmol, 1.5 equiv) and allyl bromide (174 µL, 2.012 mmol, 1.5 equiv) were added to a suspension of ethyl 3-(difluoromethyl)-1*H*-pyrazole-4-carboxylate, **1** (254.9 mg, 1.341 mmol) in dry acetone (3 mL) under nitrogen. The resulting mixture was stirred at room temperature for 4 h, then diluted with water and extracted with EtOAc. The combined organic layers were washed with brine and dried over anhydrous

122 $MgSO_4$, filtered, and concentrated under vacuum. The residue obtained was purified
123 by flash chromatography performed on silica gel 60, 230–400 mesh (Merck,
124 Darmstadt, Germany) , using hexane/EtOAc mixtures from 9:1 to 8:2 as eluent, to give
125 allyl derivative **2** as an oil (216.3 mg, 70%).

126 *Synthesis of 1-allyl-3-(difluoromethyl)-1H-pyrazole-4-carboxylic acid*, **3**. A solution of
127 ethyl ester **2** (215.2 mg, 0.934 mmol) in a mixture of 1 M aqueous NaOH (4 mL) and
128 EtOH (3 mL) was stirred at room temperature for 2 h (reaction monitored by thin layer
129 chromatography on F254 silica gel plates, hexane/EtOAc 1:1). The reaction mixture
130 was diluted with water, acidified to pH 3-5 with citric acid, and extracted with EtOAc.
131 The combined organic extracts were washed with brine and dried over anhydrous
132 $MgSO_4$, filtered, and concentrated under vacuum to afford acid **3** as a white solid
133 which was used in the next step without further purification (186.6 mg, 98.7%).

134 *Synthesis of 1-allyl-3-(difluoromethyl)-N-(3',4',5'-trifluoro-[1,1'-biphenyl]-2-yl)-1H-*
135 *pyrazole-4-carboxamide*, **5**. A suspension of acid **3** (186.6 mg, 0.923 mmol) and PCl_5
136 (207.6 mg, 0.997 mmol, 1.08 equiv) in anhydrous Et_2O (4.5 mL) was stirred at room
137 temperature under nitrogen atmosphere until complete formation of the acid chloride
138 intermediate (about 1h, reaction monitored by thin layer chromatography on F254
139 silica gel plates, hexane/EtOAc 1:3). The solvent was evaporated under vacuum and
140 the residue was dissolved in anhydrous THF (1 mL) and added via cannula to a solution
141 of anhydrous pyridine (150 µL, 1.846 mmol, 2 equiv) and 2-aminobiphenyl **4** (206.1 mg,
142 0.923 mmol, 1 equiv) in THF (1 mL) at 0 ^{o}C under nitrogen. The resulting mixture was
143 stirred at room temperature for 1.5 h, then diluted with water, acidified to pH 3–4
144 with citric acid and extracted with EtOAc. The organic extracts were washed with a 5%

aqueous solution of $NaHCO_3$ and brine, dried over anhydrous $MgSO_4$, filtered, and concentrated under vacuum. The residue obtained was purified by chromatography, using hexane/EtOAc mixtures from 9:1 to 6:4 as eluent, to give, in order of elution, unreacted 2-aminobiphenyl **4** (10.1 mg, 4.9%) and amide **5** (324.7 mg, 90.7% based on recovered starting material) as a white solid.

Synthesis of 6-(3-(difluoromethyl)-4-((3',4',5'-trifluoro-[1,1'-biphenyl]-2-yl)carbamoyl)-1H-pyrazol-1-yl)hex-4-enoic acid, **6**. But-3-enoic acid (52.8 mg, 52.2 µL, 0.615 mmol, 3 equiv) was added to a suspension of **5** (83.4 mg, 0.205 mmol), 2nd Generation Grubbs Catalyst [(1,3-bis(2,4,6-trimethylphenyl)-2-imidazolidinylidene)dichloro(phenylmethylene)(tricyclohexylphosphine) ruthenium] (10.5 mg, 0.012 mmol, 0.06 equiv) and CuI (3.0 mg, 0.016 mol, 0.08 equiv) in anhydrous Et_2O (2.4 mL) under nitrogen. The resulting mixture was stirred at 60 ^oC for 6 h under microwave irradiation (300 W). After this time, the solvent was evaporated under vacuum and the residue obtained was purified by chromatography. Elution, first with CH_2Cl_2 and then with CH_2Cl_2-Et_2O 9:1, gave unreacted starting material **5** (24.8 mg). Further elution, first with $CHCl_3$ and then with $CHCl_3$/MeOH 9:1, afforded the corresponding cross-metathesis product **6** (44.8 mg, 66.8% based on recovered starting material) as an approximately 8:2 mixture of *E*/*Z* geometrical isomers.

Synthesis of 6-(3-(difluoromethyl)-4-((3',4',5'-trifluoro-[1,1'-biphenyl]-2-yl)carbamoyl)-1H-pyrazol-1-yl)hexanoic acid (Hapten FX*n*). A solution of geometric isomers of alkene **6** (37.3 mg, 0.08014 mmol) in a mixture of 10% Pd/C (20 mg) in EtOAc (1.9 mL) was hydrogenated under a hydrogen pressure of 3 atm for 4 h. The black suspension was filtered through a pad of Celite with EtOAc, the filtrate was

concentrated in vacuum, and the residue was purified by chromatography, using $CHCl_3$/MeOH 95:5 as eluent, to give hapten FX*n* (29.6 mg, 79%) as a white solid, m.p. 132.6–133.8 ^{o}C (crystals obtained from slow evaporation from $CHCl_3$ solution). IR (neat) ν_{max} (cm^{-1}): 3500—2500 (br, s), 3423 (w), 3257 (m), 3127 (w), 2952 (w), 1710 (m), 1654 (m), 1617 (m), 1545 (s), 1045 (s), 760 (s). ^{1}H-NMR (300 MHz, $CDCl_3$) δ (ppm): 8.16 (1H, d, *J* = 8.2 Hz, H-3 BiPh), 8.04 (1H ,s, H-5 Pz), 7.86 (1H, br t, *J* = 3.5 Hz, NH), 7.50–7.36 (1H, m, H-4 BiPh), 7.25–7.19 (2H, m, H-5 and H-6 BiPh), 7.06–6.94 (2H, m, H-2′ and H-6′ BiPh), 6.65 (1H, t, *J* = 54.2 Hz, CHF_2), 4.13 (2H, d, *J* = 7.1 Hz, H-5), 2.39 (2H, t, *J* = 7.2 Hz, H-2), 2.00-1.86 (2H, m, H-4), 1.74-1.56 (2H, m, H-3). ^{19}F-NMR (282 MHz, $CDCl_3$) δ (ppm): −109.0 (2F, s, CHF_2), −134.2 (2F, d, *J* = 20.6 Hz, F-3′ and F-5′ BiPh), −162.0 (1F, t, *J* = 20.6 Hz, F-4′ BiPh). ^{13}C-NMR (75 MHz, CD_3D) δ (ppm) 176.9 (CO_2H), 163.1 (CONH), 152.2 (ddd, *J* = 248.8, 10.2, 4.5 Hz, C-3′ and C-5′ BiPh), 147.0 (t, *J* = 24.7 Hz, C-3 Pz), 140.3 (dt, *J* = 250.0, 15.4 Hz, C-4′ BiPh), 137.3 (td, *J* = 8.5, 5.1 Hz, C-1′ BiPh), 137.0 (C-2 BiPh), 135.2 (C-1 BiPh), 133.4 (C-5 Pz), 131.4 (C-4 BiPh), 130.2 (C-5 BiPh), 128.8 (C-3 BiPh), 128.4 (C-6 BiPh), 116.7 (C-4 Pz), 114.3 (dd, *J* = 14.6, 6.8 Hz, C-2′ and C-6′ BiPh), 111.2 (t, *J* = 235.1 Hz, CHF_2), 53.5 (C-5), 34.1 (C-2), 30.4 (C-4), 22.9 (C-3). HRMS (TOF MS ES+): *m/z* calcd for $C_{22}H_{19}F_5N_3O_3$ $[M+H]^+$ 468.1341, found 468.1340.

Preparation of Hapten FX*c*. The schematic representation of the synthesis of hapten FX*c* is depicted in Figure 3.

Synthesis of 3-(dibromomethyl)-1-methyl-N-(3′,4′,5'-trifluoro-[1,1'-biphenyl]-2-yl)-1H-pyrazole-4-carboxamide, **7**. A solution 1 M of BBr_3 in CH_2Cl_2 (3.15 mL, 3.15 mmol, 6 equiv) was dropwise added to a solution of fluxapyroxad (200 mg, 0.525 mmol) in anhydrous CH_2Cl_2 (8 mL) at −78 ^{o}C under nitrogen. The reaction mixture was allowed to

warm slowly to room temperature and was stirred for 4 h. The mixture was then cooled to 0 ^{o}C, then carefully quenched with water and extracted with CH_2Cl_2. The combined organic extracts were washed with brine, dried over anhydrous $MgSO_4$, and concentrated under reduced pressure to give dibromide **7** (251 mg, 95.1%) as a solid, which was deemed sufficiently pure to be used in the next step without any further purification.

Synthesis of 3-formyl-1-methyl-N-(3',4',5'-trifluoro-[1,1'-biphenyl]-2-yl)-1H-pyrazole-4-carboxamide, **8**. A solution of dibromide **7** (182 mg, 0.362 mmol) in anhydrous pyridine (500 µL) was stirred at 100 ^{o}C under nitrogen for 90 min. The resulting mixture was cooled and poured into ice-cold water and extracted with EtOAc, the combined organic layers were washed with water and brine and dried over anhydrous $MgSO_4$. Evaporation of the solvent under reduced pressure and filtration through a small plug of silica gel, washing with CH_2Cl_2/EtOAc 9:1, afforded nearly pure aldehyde **8** (118.5 mg, 91%).

Synthesis of (E/Z)-6-(1-methyl-4-((3',4',5'-trifluoro-[1,1'-biphenyl]-2-yl)carbamoyl)-1H-pyrazol-3-yl)hex-5-enoic acid, **9**. A suspension of 5-(triphenylphosphonio)pentanoic acid bromide (144.5 mg, 0.326 mmol, 2 equiv) in anhydrous hexamethylphosphoramide (442 µL) and THF (600 µL) was sonicated at room temperature under nitrogen until a clear solution was obtained. Then, a 0.5 M solution of potassium hexamethyldisilazide in toluene (1.32 mL, 0.660 mmol, 4 equiv) was dropwise added under the same conditions to obtain a deep orange solution of the corresponding Wittig reagent (ylide). The above mixture was cooled with stirring in an ice-water bath, and then a solution of aldehyde **8** (58.6 mg, 0.163) in anhydrous THF

(600 µL) was added via cannula. After 1.5 h, the reaction mixture was poured into ice-water, acidified to pH 2–3 with 1 M HCl and extracted with EtOAc. The combined organic layers were washed with a saturated aqueous solution of LiCl and brine, dried over anhydrous $MgSO_4$, and concentrated under reduced pressure to give an oily residue (193 mg) that was purified by chromatography, using $CHCl_3$/MeOH mixtures from 100:0 to 95:5 as eluent, to afford alkene **9** (49.2 mg, 68%) as a thick oil. According to its ^{1}H-NMR spectrum, it was a *ca*. 3:2 mixture of *E*- and *Z*-isomers of the double bond.

Synthesis of 6-(1-methyl-4-((3',4',5'-trifluoro-[1,1'-biphenyl]-2-yl)carbamoyl)-1H-pyrazol-3-yl) hexanoic acid (Hapten FX*c*). To a solution of **9** (44.8 mg, 0.101 mmol) in EtOAc (2 ml) was added Pd/C (10%, 23 mg) and the mixture was hydrogenated at 3 atm for 4.5 h. The catalyst was filtered through a pad of silica gel and the filter cake was washed with EtOAc. The combined filtrates were concentrated under vacuum to give hapten FX*c* (41.2 mg, 92.6%) as a white solid, m.p. 104.4–105.8 ^{o}C (from hexane/EtOAc/$CHCl_3$). IR (neat) ν_{max} (cm^{-1}): 2500–3350 (br, s), 3331 (s), 3122 (w), 3073 (w), 2945 (m), 1741 (s), 1697 (s), 1670 (s), 1615 (s), 1532 (s), 1489 (s), 1425 (s), 1207 (s), 1038 (s), 760 (s). ^{1}H-NMR (300 MHz, $CDCl_3$) δ (ppm): 8.20 (1H, d, *J* = 8.2 Hz, H-3 BiPh), 7.50 (1H, s, H-5 Pz), 7.48-7.36 (1H, m, H-4 BiPh), 7.25–7.12 (3H, m, H-5 and H-6 BiPh and NH), 7.12–6.93 (2H, m, H-2' and H-6' BiPh), 3.83 (3H, s, NMe), 2.67 (2H, t, *J* = 7.5, H-6), 2.33 (2H, t, 2H, t, *J* = 7.5, H-2), 1.64 (4H, quint, *J* = 7.5, H-3 and H-5), 1.35 (2H, m, H-4). ^{19}F-NMR (282 MHz, $CDCl_3$) δ (ppm): −133.0 (2F, d, *J* = 20.6 Hz, F-3' and F-5' BiPh), −160.9 (1F, t, *J* = 20.6 Hz, F-4' BiPh). ^{13}C-NMR (75 MHz, $CDCl_3$) δ (ppm): 178.6 (CO_2H), 161.4 (CONH), 153.0 (C-3 Pz), 151.6 (ddd, *J* = 252.3, 10.0, 4.1 Hz, C-3' and C-5' BiPh), 139.5 (dt, *J* = 253.7, 14.9 Hz, C-4' BiPh), 134.5 (td, *J* = 7.7, 5.0 Hz, C-1' BiPh),

134.7 (C-2 BiPh), 131.9 (C-5 Pz), 130.2 (C-1 BiPh), 131.1 (C-1 BiPh), 130.1 (C-6 BiPh), 129.7 (C-4 BiPh), 125.0 (C-5 BiPh), 123.2 (C-3 BiPh), 115.4 (C-4 Pz), 113.8 (dd, *J* = 14.6, 6.8 Hz, C-2′ and C-6′ BiPh), 39.2 (NMe), 33.9 (C-2), 28.9 (C-4), 28.2 (C-6), 27.5 (C-5), 24.5 (C-3). HRMS (TOF MS ES+): *m/z* calcd for $C_{23}H_{23}F_3N_3O_2$ $[M+H]^+$ 446.1686, found 446.1681.

Preparation of Protein Conjugates. The synthesis of *N*-hydroxysuccinimidyl esters of haptens FX*n* and FX*c* (Figures 2 and 3) was performed by reaction of the purified hapten with *N,N′*-disuccinimidyl carbonate and Et_3N in anhydrous CH_3CN. Both haptens were covalently linked to BSA, OVA, and HRP using a 24, 8, and 5.5 molar excess, respectively, of the corresponding purified *N*-hydroxysuccinimidyl ester according to previously established procedures.[22] The obtained hapten-to-protein molar ratio (MR) of the prepared conjugates was determined by MALDI-TOF mass spectrometry.

Antibody Generation. Procedures for animal immunization were approved by the Ethics Committee of the University of Valencia for Animal Experimentation and Welfare. Animal manipulation was carried out according to Spanish and European laws and guidelines concerning the protection of animals used for scientific purposes (RD 1201/2005, Law 32/2007, and European Directive 2010/63/EU). Two white female rabbits of around 2 kg each were subcutaneously immunized with 300 μg of BSA–FX*n* or BSA–FX*c* conjugate in a 1:1 emulsion between phosphate buffer solution and Freund's adjuvant (complete for the first injection and incomplete for further boosts). The immunogen was given 4 times with 21-day intervals. Whole blood was taken 10 d after the fourth injection and it was allowed to coagulate overnight at 4 °C. The serum

was separated by centrifugation and antibodies were precipitated twice with 1 volume of a saturated ammonium sulfate solution. Precipitated antisera were stored at 4 °C, and a working antibody solution was prepared by 4-fold dilution with PBS containing 1% (w/v) BSA and kept at 4 °C.

Immunoassay Development. Immunoassays were developed using the antibody-coated direct format and the conjugate-coated indirect format according to previously published procedures.[23] Starting from a 10 µM fluxapyroxad solution in PBS, eight-point fluxapyroxad standard curves, including a blank, were prepared by 10-fold serial dilution in PBS. A 10 mM fluxapyroxad stock solution in anhydrous *N*,*N*-dimethylformamide was used to prepare the first standard solution. For sample analysis, fruit juices were purchased from local supermarkets. The competitive reaction was carried out with samples diluted in MilliQ water and the corresponding immunoreagent in 2×PBS containing 0.05% (v/v) Tween 20. In order to ascertain the minimum sample dilution to obtain a reliable determination of fluxapyroxad, the analytical parameters of the standard curves prepared in the diluted matrices were compared to those from curves run in water. Recovery studies with fluxapyroxad fortified fruit samples were performed in order to examine the accuracy and precision of the developed immunoassays. Each sample was analyzed in duplicate wells and average absorbance values were interpolated into the standard curve run in triplicate wells in the same plate.

Data Treatment. Microplates were read at 492 nm using 650 nm as reference wavelength with a PowerWave HT instrument (BioTek, Winooski, VT). Experimental values were fitted to a four-parameter logistic equation using the SigmaPlot software

package from SPSS Inc. (Chicago, IL). Assay sensitivity was defined as the concentration of analyte at the inflection point of the fitted curve. This value typically corresponds to a 50% reduction (IC_{50}) of the maximum absorbance (A_{max}). The limit of detection (LOD) and the dynamic range of the assay were estimated as the concentration of analyte providing a decrease of A_{max} of 10% and between 20% and 90%, respectively. Cross-reactivity was determined as the percentage of the quotient between the IC_{50} value for fluxapyroxad and the IC_{50} value for the evaluated compound.

Sample Preparation. Peach, apple, and grape juices were obtained from local supermarkets. In order to obtain contaminated samples, grapevines were treated with a commercial formulation kindly provided by BASF Crop Protection (Sercadis, fluxapyroxad 300 g/L) at the dose recommended by the manufacturer using a manual sprayer. Three days after the treatment, grape samples (500 g) were harvested, and upon arrival at the laboratory berries were separated from the stems, homogenized using a T-25 Ultra-Turrax blender (IKA, Staufen, Germany), and stored in polypropylene tubes at −20 °C until analysis. The QuEChERS method was employed for extraction of fungicide residues from samples.[24] Briefly, homogenized grapes (5 g) were introduced into 50 mL polypropylene centrifuge tubes containing 0.5 g of sodium acetate and 2.0 g of anhydrous magnesium sulfate. Samples were then extracted by vortexing for 1 min with 5 mL of 1% (v/v) acetic acid in acetonitrile containing 500 µg/L of triphenylphosphate as internal standard, and centrifuged for 5 min at 2200×g. The extracts (1 mL) were then cleaned-up with 50 mg of PSA (primary–secondary amine) and 150 mg of anhydrous magnesium sulfate, vortexed for 1 min, and centrifuged 5 min at 6700×g. Purified extracts were then filtered through a 0.22 µm Teflon filter and

307 analyzed by the optimized cELISA and by ultra-performance liquid chromatography
308 coupled to a triple quadrupole tandem mass spectrometer (UPLC–MS/MS).

309 **Chromatographic analysis.** Determinations were carried with an Acquity UPLC
310 system (Waters, Milford, MA) equipped with a 50 mm × 2.1 mm i.d., 1.7 µm, Bridged
311 Ethylene Hybrid C18 column. The injection volume was 5 µL and the mobile phase
312 consisted of 0.5% (v/v) formic acid in water (A) and acetonitrile (B). The elution
313 gradient started at 50% of B at a flow-rate of 400 µL/min, and then B was linearly
314 increased to 95% in 4 min. The obtained retention times, using the aforementioned
315 conditions, were 1.36 and 2.36 min for fluxapyroxad and triphenylphosphine as
316 internal standard, respectively. Tandem mass acquisitions were performed in a Waters
317 Acquity triple quadrupole mass spectrometry detector, equipped with a Z-spray
318 electrospray ionization source, with 3.5 kV capillary voltage, at 120 °C as source
319 temperature, and 300 °C as desolvation temperature. The employed parameters were:
320 ESI+, parent ion *m/z* 382.0, daughter ions *m/z* 314.3 and 342.3, 30 eV collision energy,
321 and 20 V cone energy for fluxapyroxad; and ESI+, parent ion *m/z* 328.3, daughter ions
322 *m/z* 77.0 and 152.4, 30 eV collision energy, and 25 V cone energy for
323 triphenylphosphine.

324 **RESULTS AND DISCUSSION**

325 **Hapten Synthesis.** Two functionalized haptens mimicking fluxapyroxad were
326 designed, namely haptens FX*n* and FX*c* (Figures 2 and 3). Both haptens incorporate a
327 spacer arm suitable for protein conjugation, namely a carboxylated hydrocarbon linear
328 chain, in complementary positions of the pyrazole ring. The spacer arm in hapten FX*n*
329 was a substituent of the methyl group at N-1 which would produce an insignificant

effect on the electronic distribution and conformational mobility of the fluoxapyroxad framework. In the case of hapten FX*c*, the incorporation of the alkyl chain as a C-3 substituent of the difluoromethyl group would entail a greater change, both in the electronic distribution of the pyrazole ring and in the conformational disposition of the amide group, due to the weak electron-donor effect of the alkyl chain compared to the withdrawing electron effect of the difluoromethyl group and the greater steric hindrance introduced by the highly flexible linear hydrocarbon spacer arm.

The preparation of hapten FX*n* is outlined in Figure 2. The synthesis began with the incorporation of an allyl group at the N-1 position of ethyl 1*H*-pyrazole-4-carboxylate **1**, which was used as both a blocking group of this position during the construction of the fluxapyroxad framework and as a pivotal moiety for the subsequent elaboration of the hydrocarbon chain constituting the spacer arm. Thus, alkylation of **1** with allyl bromide under basic conditions led to allyl ether **2**, whose ethyl ester moiety was hydrolyzed under conventional basic conditions to afford pyrazole carboxylic acid **3**. Activation of the carboxylate group to the acid chloride, by treatment with phosphorus pentachloride in ether at room temperature, followed by reaction with biphenyl amine **4** in the presence of pyridine, to quench the acid (HCl) formed, led to amide **5**, thus completing the part of the hapten skeleton common with the parent analyte. Elaboration of the C5 carboxylated chain at N-1 from **5** was carried out via a microwave irradiation promoted cross-metathesis reaction of the allyl ether moiety with but-3-enoic acid catalyzed by Grubbs' second generation catalyst and copper iodide as co-catalyst. The cross-metathesis reaction afforded alkene acid **6** as a *ca*. 8:2 mixture of *E*/*Z* geometrical isomers of the double bond that was hydrogenated under heterogeneous catalyzed conditions to give the desired hapten. The synthesis of

354 hapten FX*n* was thus completed from pyrazole **1** in six synthetic steps and 31% overall
355 yield.

356 On the other hand, the synthesis of hapten FX*c* was initiated from fluxapyroxad and
357 involved the initial transformation of the difluoromethyl group into a dibromomethyl
358 group (Figure 3), which underwent hydrolysis to a formyl group by heating in pyridine
359 and subsequent aqueous workup to afford aldehyde **8**. The elaboration of the
360 carboxylated spacer arm was carried out, in this case, through a Wittig olefination
361 reaction between the aldehyde carbonyl group of **8** and the ylide derived from (4-
362 carboxybutyl)triphenylphosphonium bromide and potassium hexamethyldisilazane,
363 which afforded the olefinic acid **9** as a *ca*. 3:2 mixture of *trans* and *cis* isomers,
364 followed by catalytic hydrogenation of the generated double bond. The synthesis of
365 hapten FX*c* was thus achieved in four steps with an excellent 63% overall yield from
366 fluxapyroxad.

367 **Immunoreagent Preparation.** The same coupling procedure, the active ester
368 method, was applied for preparation of both the immunizing and the assay conjugates.
369 Previously, the carboxyl functional group of each hapten was activated under smooth
370 conditions with *N,N*'-disuccinimidyl carbonate, which allowed the easy isolation and
371 further chromatographic purification of the corresponding active esters, FX*n*-NHS and
372 FX*c*-NHS esters (Figures 2 and 3). Freshly prepared and purified succinimidyl esters of
373 haptens were used for covalently coupling to BSA, OVA, and HRP. Since the pure active
374 ester was employed, no succinimide and carbodiimide were present during the
375 coupling reaction; thus, no secondary and cross-coupling reactions could occur and
376 higher coupling yields could be accomplished, so lower amounts of hapten were

377 required. The obtained hapten-to-protein molar ratios (MR), determined by MALDI-
378 TOF mass spectrometry, for BSA, OVA, and HRP conjugates were 18.9, 5.3, and 1.2 and
379 15.2, 5.4 and 1.3 for haptens FX*n* and FX*c*, respectively (Figure 4). These values are
380 within the optimum range of MR values for immunizing and assay conjugates.

381 Four rabbits were immunized; two with conjugate BSA–FX*n* and two with conjugate
382 BSA–FX*c*. Thus, two polyclonal antibodies, named FX*n*#1 and FX*n*#2, were obtained
383 from the conjugate of hapten FX*n*, and two, named FXc#1 and FXc#2, from the
384 conjugate of hapten FX*c*.

385 **Antibody Characterization.** Specificity of the antibodies was assessed by indirect
386 cELISA using the homologous coating conjugate. All of the generated polyclonals were
387 highly specific of fluxapyroxad. Inhibition was negligible even with a concentration of
388 10 µM of other SDH-inhibitor fungicides (penthiopyrad and fluopyram) as well as other
389 widely used pesticides which may be present together with fluxapyroxad in real food
390 samples (azoxystrobin, picoxystrobin, pyraclostrobin, kresoxim-methyl, fenhexamid,
391 fluopicolide, pyrimethanil, and cyprodinil). Only a slight binding was observed with
392 boscalid. Interestingly, the cross-reactivity was 10 times lower with antibodies
393 obtained from hapten FX*n* than with FX*c*-type antibodies, indicating the relevance of
394 the linker tethering site for antibody specificity.

395 Antibody affinity was evaluated by checkerboard cELISA using homologous (same
396 hapten as that of the immunizing conjugate) and heterologous (the hapten was
397 different from the immunizing conjugate) conjugates in two different assay
398 procedures: the antibody-coated direct format and the conjugate-coated indirect
399 format (Table 1). According to homologous assays, little difference was observed

400 between the titers (antibody dilution affording an A_{max} value around 1.0 in the absence
401 of analyte in solution) of FX*n*- and FX*c*-type antibodies, and high-affinity antibodies to
402 fluxapyroxad were obtained from both of the immunizing haptens, with IC_{50} values
403 generally in the low nanomolar range. The only exception was antibody FX*c*#1 which
404 showed a high IC_{50} value in the indirect homologous assay. Therefore, the pyrazole ring
405 seems to be a suitable linker tethering site in the fluxapyroxad molecule for high-
406 affinity antibody generation. The lowest IC_{50} value using homologous conjugates was
407 obtained with antibody FX*n*#2 in both assay formats. This result, together with the
408 higher specificity of FX*n*-type antibodies, indicated a slight superiority of hapten FX*n* to
409 elicit the best immune response. This finding could be explained by the minor changes
410 introduced by the spacer arm in hapten FX*n* compared to the non-negligible changes in
411 the electronic and conformational properties of the pyrazole ring produced by the
412 linker in hapten FX*c* as discussed above.

413 It is well established that heterologous conjugates can help to improve
414 immunoassay sensitivity. However, if too drastic heterologies are used, binding of the
415 antibody to the assay conjugate could be insufficient, particularly in the antibody-
416 coated format. The two haptens (FX*n* and FX*c*) prepared in this study held the spacer
417 arm in proximal positions, so a similar orientation of the distal moieties was expected.
418 As envisioned, all of the antibodies recognized the corresponding heterologous
419 conjugate, including the enzyme tracer in the direct assay format, with the same or
420 similar titers (Table 1). The slope of the fitted inhibition curve was generally between
421 0.8 and 1.0, and the background signal was always below 0.1. Mostly, assay sensitivity
422 could be enhanced by using the heterologous conjugates; the IC_{50} values were
423 generally reduced around 50% or more if compared to the homologous assay. The

differences in the electronic and structural properties between both haptens did not seem to hinder antibody binding. These results are in accordance with the Landsteiner's principle[25] stating that the specificity of antibodies is mainly directed towards the distal moieties of small organic molecules.

The most sensitive immunoassays were found with antibody FX*n*#2 in combination with the heterologous conjugate, both in the direct and indirect assay formats. The main analytical parameters of the standard curve of the best immunoassay in each format are listed in Figure 5. Both standard curves showed A_{max} values over 1.0 and slope values close to 1.0, which constitute excellent parameters for immunoassay application. The IC_{50} for fluxapyroxad of the direct and indirect assays were 0.40 nM (0.15 µg/L) and 1.51 nM (0.58 µg/L), respectively. The calculated LOD values were 0.05 nM (0.019 µg/L) and 0.12 nM (0.046 µg/L) for the direct and indirect assay, respectively. Therefore, in this particular case, the sensitivity of the direct assay was slightly better than that of the indirect assay, probably due to the antibody characteristics. Generally, when polyclonal antibodies are used, heterologous conjugates enhance assay sensitivity of both cELISA formats, and similar sensitivities are usually attained with either format, as previously reported for other small chemical compounds.[26–28] Finally, the theoretical dynamic working range was established between 0.09 nM (0.034 µg/L) and 4.30 nM (1.640 µg/L) for the direct assay, and between 0.30 nM (0.114 µg/L) and 17.89 nM (6.820 µg/L) for the indirect format. Due to their similar sensitivities, the two optimized immunoassays were further evaluated for food sample analysis.

446 **Sample Analysis.** Matrix effects of peach, apple, and grape juices were determined
447 by running standard curves of fluxapyroxad in juices diluted in water (from 1/5 to
448 1/500). Very low interferences were seen with peach and apple juices in the direct
449 assay, whereas grape juice seemed to induce slight matrix effects (Figure S1). On the
450 other hand, similar results were found with the indirect assay, where the grape juice
451 showed the strongest interference (Figure S2). In all cases, a 1/25- or a 1/50-fold
452 dilution seemed to minimize the observed matrix effects.

453 Next, peach, apple, and grape juices were fortified with fluxapyroxad and analyzed
454 by the developed immunoassays. Table 2 lists the recovery rates and the coefficients
455 of variation found by the direct and indirect cELISA. For the former assay, precise and
456 accurate results were obtained for all of the juices in the range from 5 to 500 µg/L,
457 with just a 1/25-fold sample dilution in water. Coefficients of variation higher than 20%
458 were only seen for the lowest and the highest assayed concentration in apple and
459 grape juice, respectively. For all three types of samples, the limit of quantitation (LOQ)
460 of the direct immunoassay could be set at 5 µg/L. Regarding the indirect assay, a 1/50-
461 fold sample dilution in water was required in order to obtain accurate and precise
462 results (Table 2). Somewhat unprecise results were found with apple juice samples at
463 the lowest assayed concentrations. On the contrary, good recovery rates and
464 acceptable coefficients of variation were found for peach and grape juices spiked with
465 fluxapyroxad at concentrations between 30 and 3000 µg/L. Therefore, the LOQ of the
466 indirect immunoassay could be established at 300 µg/L for apple juice and at 30 µg/L
467 for peach and grape juices. Consequently, the LOQs displayed by the developed direct
468 and the indirect cELISAs were much lower than the maximum residue limits (MRL)
469 established for peach, apple, and grape samples by the European Commission (1500,

900, and 3000 μg/L, respectively)[29] and by the US Department of Agriculture (3000, 800, and 2000 μg/L, respectively).[30] Nevertheless, in terms of sensitivity and robustness, the direct assay seemed to be the best choice for the immunoanalysis of fluxapyroxad residues in foodstuffs.

Due to the lack of juice samples with incurred residues and in order to provide compelling evidences of the suitability of the direct cELISA for the determination of fluxapyroxad residues under real conditions, a limited number of grapevines were treated with a commercial formulation of the fungicide during the 2015/2016 season near the harvest period. Grape samples were harvested three days after the treatment with fluxapyroxad and extracted using the QuEChERS method. Extracts were directly analyzed by UPLC–MS/MS and by cELISA after 500-fold dilution in MilliQ water. Linear regression analysis indicated an acceptable correlation between the developed direct immunoassay and the reference chromatographic method (y = 1.09x + 15.12; r^2=0.963), with only a slight deviation between both sets of measurements (Figure 6).

In summary, two haptens mimicking the molecule of fluxapyroxad were prepared with the linker arm located at the same ring. Moreover, bioconjugates to different proteins were obtained with adequate yields by the active ester method using the purified activated hapten, and antibodies to fluxapyroxad were generated for the first time. A slightly higher specificity was observed for those binders that were obtained from the hapten with the spacer arm at the N-1 position of the pyrazole ring. High-affinity to fluxapyroxad was observed by cELISA, with IC_{50} values in the low nanomolar range. Two immunoassays were developed in different formats with antibody FX*n*#2 and using a heterologous conjugate. In the analysis of fruit juices, the direct assay

493 outperformed the indirect assay in terms of sensitivity and robustness, so it was
494 applied to the quantitation of grape samples with incurred residues of fluxapyroxad,
495 with satisfactory results.

496 **ASSOCIATED CONTENT**

497 **Supporting Information**

498 General experimental procedures and techniques, preparation of 3',4',5'-trifluoro-
499 [1,1'-biphenyl]-2-amine (**4**), characterization data of intermediates of the synthesis of
500 hapten FX*n*, characterization data of intermediates of the synthesis of hapten FX*c*,
501 hapten activation and characterization of active esters, preparation of bioconjugates,
502 MALDI mass spectrometry analysis of immunizing and assay bioconjugates,
503 competitive ELISA procedures, antibody specificity (Table S1), juice matrix effects over
504 the direct immunoassay (Figure S1), juice matrix effects over the indirect immunoassay
505 (Figure S2), ^{1}H-NMR spectra of haptens. This material is available free of charge via de
506 Internet at http://pubs.acs.org.

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622 **Figure legends**

623 **Figure 1.** Molecular structure of fluxapyroxad.

624 **Figure 2**. Schematic representation of the synthesis and activation of hapten FX*n*.

625 **Figure 3**. Schematic representation of the synthesis and activation of hapten FX*c*.

626 **Figure 4.** MALDI-TOF-MS spectra of proteins (blue) and the corresponding
627 conjugates with haptens FX*n* (green) and FX*c* (orange): (A) BSA conjugates; (B) OVA
628 conjugates; (C) HRP conjugates.

629 **Figure 5.** Standard curve parameters of the best immunoassays in two different
630 formats (n = 3).

631 **Figure 6.** Comparison of results obtained by the developed direct cELISA and UPLC–
632 MS/MS for fluxapyroxad analysis in grapes from in-field treated vineyards. Data are
633 from four independent determinations.

Table 1. Checkerboard Screening Assay of Fluxapyroxad Antibodies (n=3)

	direct format						indirect format					
antibody	antibody titer	HRP tracer	[T][a] (ng/mL)	A_{max}	slope	IC_{50} (nM)	antibody titer	OVA coating	[C][b] (ng/mL)	A_{max}	slope	IC_{50} (nM)
FX*n*#1	10^4	FX*n*	30	2.19	1.00	10.6	10^5	FX*n*	100	1.81	0.74	6.8
	10^4	FX*c*	30	1.13	0.67	2.2	10^5	FX*c*	100	1.34	0.84	18.5
FX*n*#2	3×10^4	FX*n*	30	1.93	0.81	0.9	10^5	FX*n*	100	1.13	0.81	4.0
	3×10^4	FX*c*	30	1.14	0.93	0.4	3×10^4	FX*c*	100	1.38	0.89	1.5
FX*c*#1	10^4	FX*n*	100	0.85	0.80	4.9	10^4	FX*n*	100	1.28	0.81	8.8
	10^4	FX*c*	100	1.43	0.75	5.6	10^5	FX*c*	100	1.30	0.60	141.6
FX*c*#2	10^4	FX*n*	30	1.39	0.89	1.1	3×10^4	FX*n*	100	1.13	0.65	2.6
	3×10^4	FX*c*	30	1.35	0.62	1.9	10^5	FX*c*	100	0.88	0.62	11.7

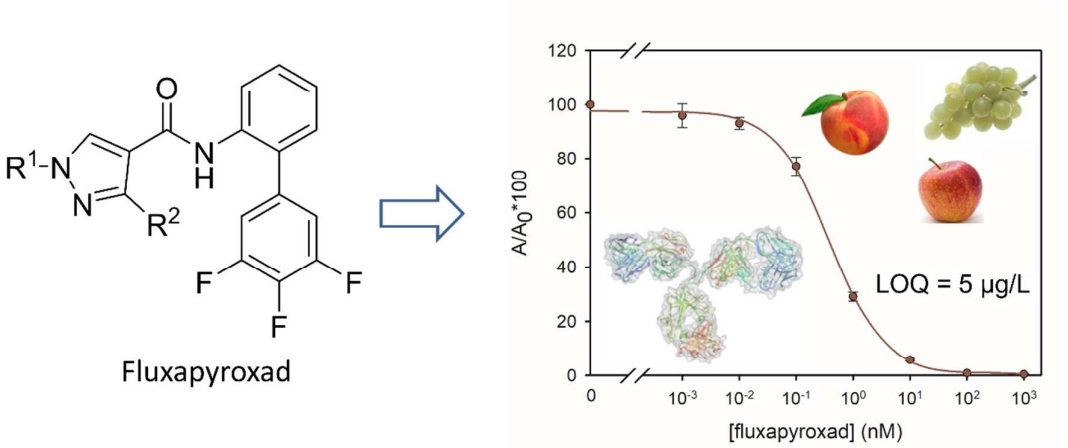
[a] Tracer concentration. [b] Coating conjugate concentration.

Table 2. Recovery Values from Spiked Fruit Juices Using the Two Developed Immunoassays (n = 5).

		peach		apple		grape	
	[FX][a]	recovered	CV	recovered	CV	recovered	CV
immunoassay	(μg/L)	(%)	(%)	(%)	(%)	(%)	(%)
direct[b]	5	118.3	18.2	89.2	31.5	110.9	12.2
	10	111.0	19.9	90.7	16.8	98.9	20.8
	20	102.8	12.1	95.5	12.7	97.4	16.0
	50	97.4	6.5	90.5	4.5	87.3	17.4
	100	92.1	11.0	85.2	9.9	86.8	11.9
	200	86.9	16.0	81.5	4.8	80.5	2.9
	500	90.7	10.7	92.5	11.8	107.8	30.5
indirect[c]	30	127.7	18.9	101.4	43.3	114.2	28.7
	100	116.0	19.2	101.2	34.0	114.0	19.3
	300	112.3	7.7	112.4	11.7	98.4	15.4
	500	100.3	4.0	102.0	6.3	91.8	16.9
	1000	98.0	8.4	91.4	5.7	85.2	14.5
	3000	93.7	6.3	95.4	15.3	78.6	22.7

[a] Spiked fluxapyroxad concentration. [b] Samples were 25-fold diluted in MilliQ water. [c] Samples were 50-fold diluted in MilliQ water.

Table of Contents Graphic



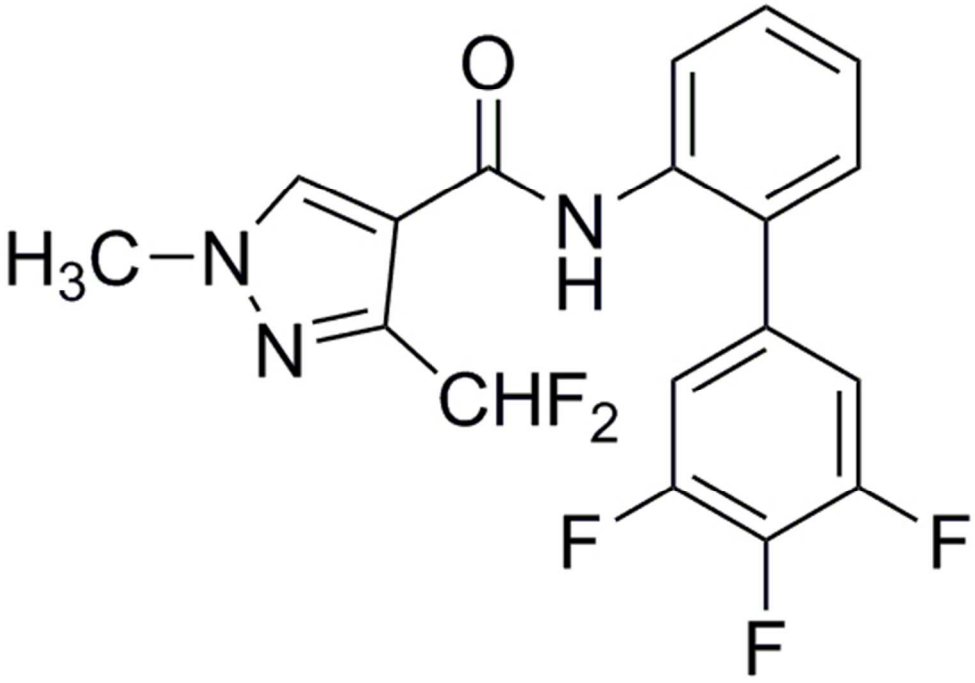


Figure 1. Molecular structure of fluxapyroxad.

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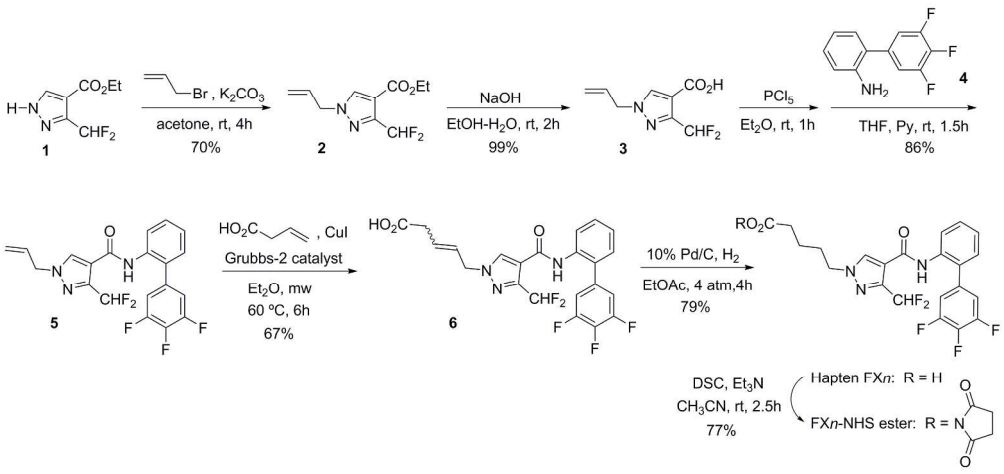

Figure 2. Schematic representation of the synthesis and activation of hapten FXn.

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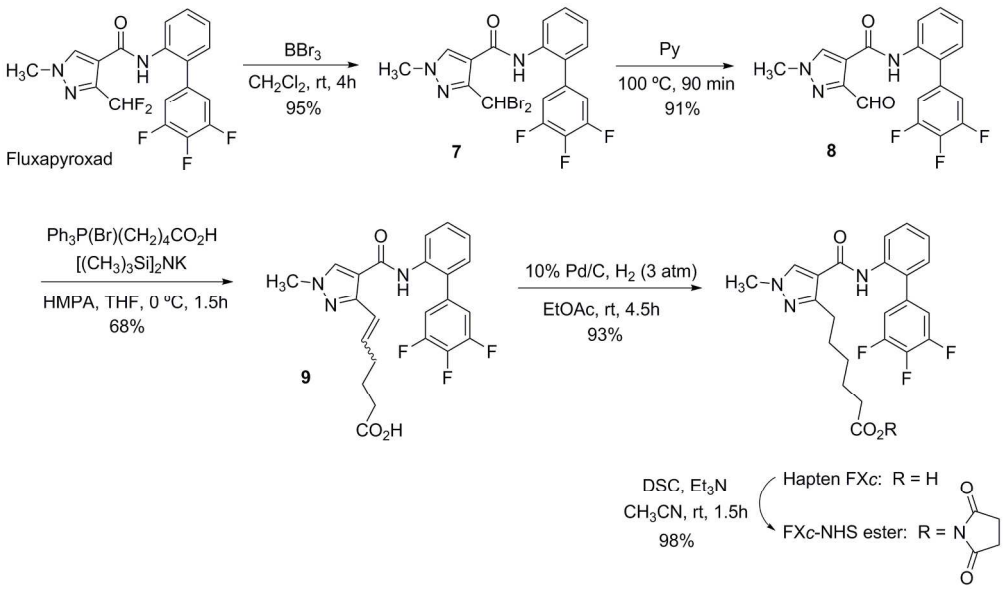

Figure 3. Schematic representation of the synthesis and activation of hapten FXc

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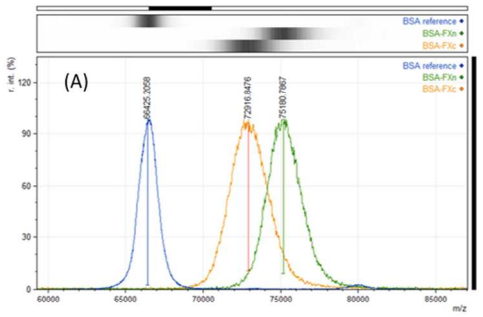
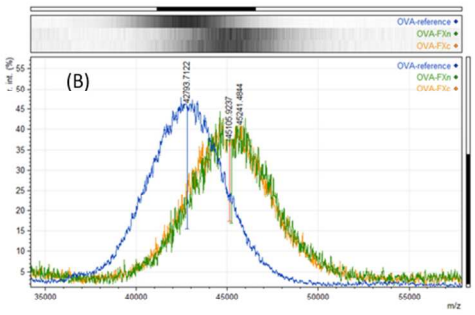
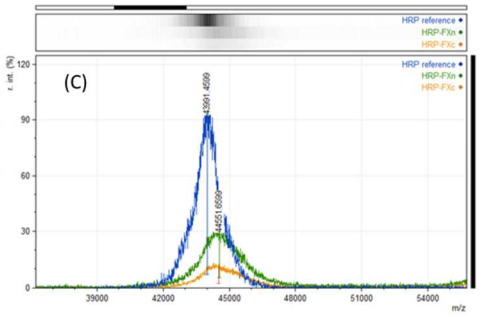


	m/z	Δ*m*/hapten	Δ(*m/z*)	MR
BSA	66425	-	-	-
BSA-FX*n*	75181	463.1	8756	18.9
BSA-FX*c*	72917	427.2	6494	15.2



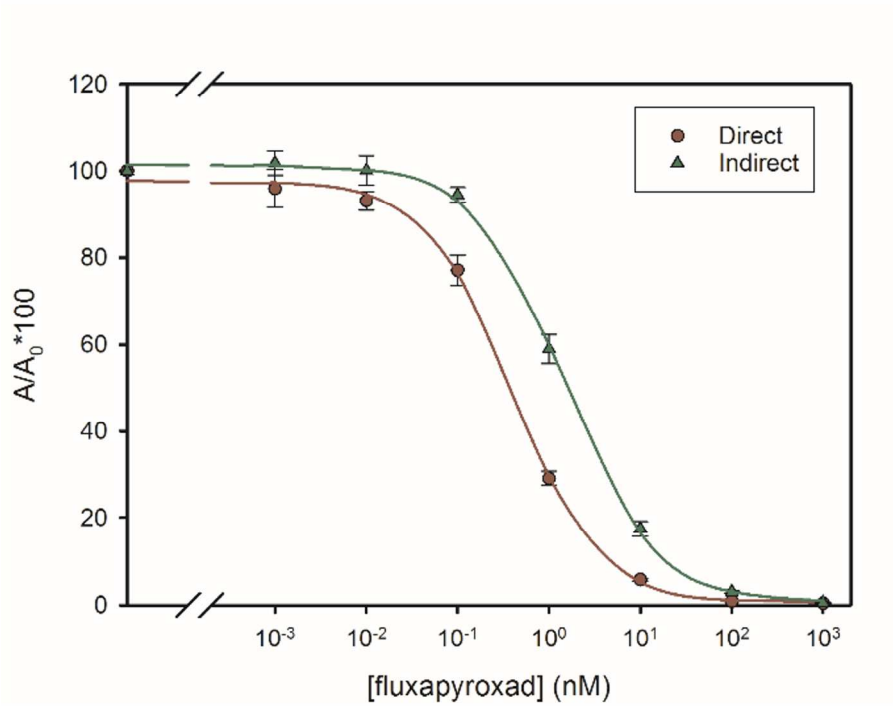
	m/z	Δ*m*/hapten	Δ(*m/z*)	MR
OVA	42794	-	-	-
OVA-FX*n*	45241	463.1	2447	5.3
OVA-FX*c*	45106	427.2	2312	5.4



	m/z	Δ*m*/hapten	Δ(*m/z*)	MR
HRP	43991	-	-	-
HRP-FX*n*	44552	463.1	561	1.2
HRP-FX*c*	44536	427.2	545¤	1.3

Figure 4. MALDI-TOF-MS spectra of proteins (blue) and the corresponding conjugates with haptens FXn (green) and FXc (orange): (A) BSA conjugates; (B) OVA conjugates; (C) HRP conjugates.

187x156mm (150 x 150 DPI)



	direct format	indirect format
antibody	FXn#2	FXn#2
(titer)	(3×10^4)	(3×10^4)
conjugate	HRP–FXc	OVA–FXc
	30 ng/mL	100 ng/mL
A_{max}	1.14 ± 0.16	1.38 ± 0.25
IC_{50} (nM)	0.40 ± 0.07	1.51 ± 0.21
slope	0.93 ± 0.11	0.89 ± 0.02
A_{min}	0.006 ± 0.007	0.009 ± 0.003

Figure 5. Standard curve parameters of the best immunoassays in two different formats (n = 3).

319x420mm (150 x 150 DPI)

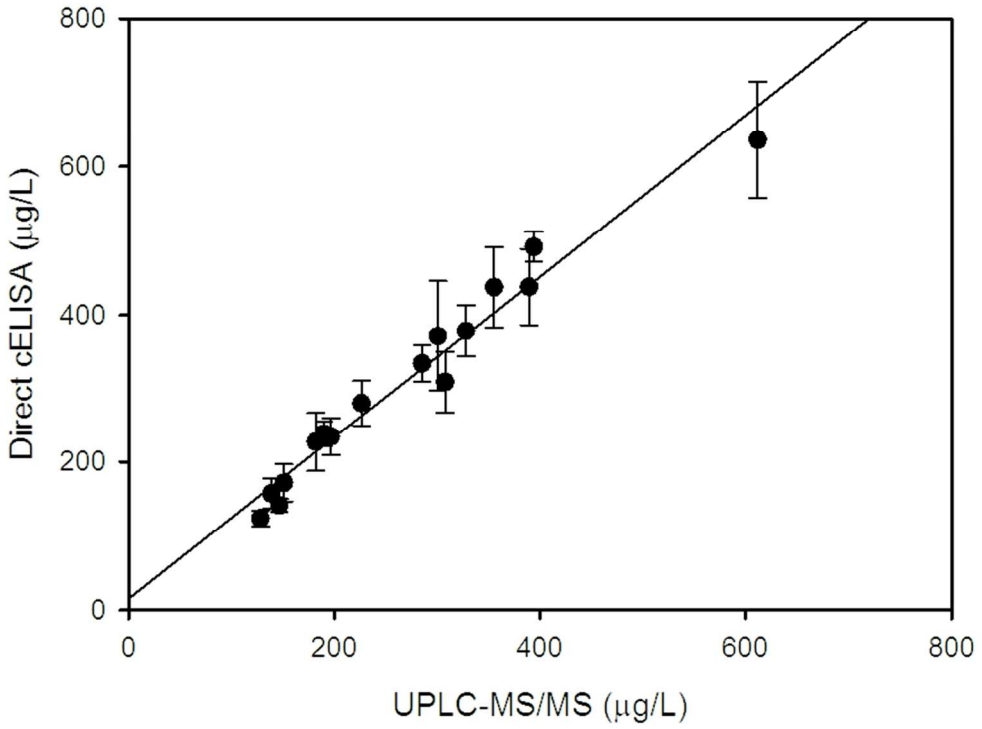


Figure 6. Comparison of results obtained by the developed direct cELISA and UPLC–MS/MS for fluxapyroxad analysis in grapes from in-field treated vineyards. Data are from four independent determinations.

151x120mm (150 x 150 DPI)